Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com

Chemical, physical, and *in vitro* characterization of research cigarettes containing denicotinized tobacco

Bonnie G. Coffa,[a, *] Christopher R.E. Coggins,[b] Michael S. Werley,[a] Michael J. Oldham,[a] Marc W. Fariss [a]

[a] *Altria Client Services LLC, Richmond, VA, USA*
[b] *Carson Watts Consulting, King, NC, USA*

ARTICLE INFO

Article history:
Received 19 January 2016
Received in revised form 4 May 2016
Accepted 11 May 2016
Available online xxx

Keywords:
Very low nicotine tobacco
Reduced nicotine content cigarettes
Denicotinized tobacco
Denic
Tobacco constituents

ABSTRACT

The use of very low nicotine tobacco cigarettes is currently being investigated as a possible harm reduction strategy. Here, we report the smoke chemistry, toxicity, and physical characteristics of very low nicotine cigarettes that were made using blended tobacco processed through a supercritical CO_2 fluid extraction, which resulted in elimination of 96% of nicotine content (denicotinized (denic) tobacco). Three types of test cigarettes (TCs) were manufactured with tobacco filler containing 100% denic tobacco (TC100), 50% denic tobacco and 50% unextracted tobacco (TC50/50), and 100% unextracted tobacco (TC0). Mainstream smoke (MS) was generated for measurement of 46 analytes and cytotoxicity and mutagenicity determination. Analysis of physical characteristics of TCs demonstrated they were well made with <5% variability among cigarettes for most parameters measured. We observed significant changes in the levels of smoke constituents, including decreases in formaldehyde, nitrosamines, and phenol, and increases in aliphatic hydrocarbons, aliphatic nitrogen compounds, aromatic amines, halogen compounds, and metals. Use of denic tobacco resulted in changes in the chemical composition of MS, but these changes did not modify biological activity as measured in the mutagenicity and cytotoxicity assays.

1. Introduction

The majority of published studies investigating the effect of varying nicotine levels in cigarettes on smoking behavior have used two types of reduced nicotine cigarettes: Quest® cigarettes [e.g., Hatsukami et al. (2010)] and Phillip Morris USA (PM USA) denicotinized (denic) tobacco research cigarettes (RCs) [e.g., Benowitz et al. (2007)]. Based on findings from these and other studies, investigators hypothesize that a 90–95% reduction in the nicotine content of cigarettes may have a significant effect on smoking cessation and dependence potential (Sherer and Lee, 2014) without increasing exposure to tobacco toxicants. These findings have sparked considerable scientific interest in the potential use of low nicotine tobacco cigarettes as a harm reduction strategy and highlight the need for additional research (Hatsukami et al., 2013).

The physical characteristics and chemical composition of the smoke of commercial tobacco cigarettes and reference research cigarettes are well documented (Bodnar et al., 2012; Counts et al., 2006; Roemer et al., 2004). In contrast, there is limited information regarding the physical design and smoke chemistry of very low nicotine (VLN) tobacco RCs. The chemical composition and physical characteristics of cigarettes have been reported to influence both the yield of smoke toxicants (Adam et al., 2010; O'Connor et al., 2008; Stephens, 2007) and tobacco use behavior (Strasser et al., 2006; Scherer, 1999).

In recent years, researchers have been unable to further their understanding of the effect of decreasing nicotine levels in cigarettes on smoking behavior as VLN cigarettes were not commercially viable and thus, are no longer available. To address this deficit, the National Institute of Drug Abuse (NIDA) announced in 2013 and 2014, the availability of SPECTRUM® reduced nicotine RCs with varying levels of nicotine (http://grants.nih.gov/grants/guide/notice-files/NOT-DA-14-004.html). Due to the renewed interest in reduced nicotine cigarettes and a potential nicotine product standard, we are publishing the results of two independent studies conducted in 2001 and 2005 on smoke chemistry and *in vitro* toxicology of PM USA denic RCs. These studies were conducted to characterize the RCs before making them available to external investigators in the public health community (Domino et al., 2013; Benowitz et al., 2007, 2012).

The objective of the research studies was to measure physical characteristics, selected smoke constituents, and two toxicological endpoints (cytotoxicity and mutagenicity) of PM USA denic tobacco

Abbreviations: ANOVA, analysis of variance; CO_2, carbon dioxide; CO, carbon monoxide; FDA, US Food and Drug Administration; GC, gas chromatography; GMO, genetically modified; GVP, gas vapor phase; HCN, hydrogen cyanide; HPHC, harmful and potentially harmful constituent; IITRI, IIT Research Institute; INBIFO, Institut fur Biologische Forschung; ISO, International Organization for Standardization; MDD, minimum detectable difference; MS, mainstream smoke; NIDA, National Institute of Drug Abuse; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*-nitrosonornicotine; NO_x, nitrogen oxides; NR, neutral red; PAHs, polyaromatic hydrocarbons; PM USA, Phillip Morris USA; RC, research cigarette; RTD, resistance to draw; TC0, 100% unextracted tobacco; TC100, 100% denicotinized tobacco; TC50/50, 50% denicotinized tobacco and 50% unextracted tobacco; TC, test research cigarette; TPM, total particulate matter; TSNAs, tobacco specific nitrosamines; VLN, very low nicotine

* Corresponding author. Regulatory Affairs, Altria Client Services LLC, 601 East Jackson Street, Richmond, VA 23261, USA.
Email address: bonnie.g.coffa@altria.com, SciencePublications@altria.com (B.G. Coffa)

http://dx.doi.org/10.1016/j.yrtph.2016.05.016
0273-2300/

RCs. In addition, comparisons of PM USA denic RCs are made to the available published literature on Quest® and SPECTRUM® reduced nicotine content cigarettes.

2. Methods

Physical measurements and cytotoxicity and mutagenicity testing of test research cigarettes (TCs) were conducted at PM USA Research and Development Product Testing Laboratory and at Philip Morris Research Laboratory, previously called Institut fur Biologische Forschung (INBIFO). TCs were also submitted to an independent research laboratory, IIT Research Institute (IITRI, Chicago, IL), for mainstream smoke (MS) chemistry, cytotoxicity and mutagenicity testing. Assays performed were in accordance with Good Laboratory Practice guidelines (U.S. Department of Health and Human Services, 1996). There were slight differences in the test methods between the two testing laboratories (differences are noted in the supplementary material), but no substantial effect on the overall results was observed. Results are reported on a per mg TPM basis; results reported on a per mg tar basis and per cigarette basis are provided in the supplementary material.

2.1. Tobacco denicotinization process

The denicotinization process used a supercritical carbon dioxide extraction procedure (Prasad and Grubbs, 1996). Briefly, a nicotine-free carbon dioxide (CO_2) solvent in the supercritical or liquid state was fed into an extraction flow system containing tobacco, and a nicotine-rich solvent was discharged from another end of the extraction flow system. Nicotine was then entrapped from the extracted supercooled CO_2 fluid and discarded. The extracted (denic) tobacco was used in the construction of the TCs.

2.2. Cigarette construction and smoking

Three types of TCs were manufactured in 2000 (tested at INBIFO) and 2004 (tested at IITRI) with tobacco filler containing 100% denic tobacco (TC100), 50% denic tobacco and 50% unextracted tobacco (TC50/50), and 100% unextracted tobacco (TC0, the control). Nicotine content (dry weight) was approximately 2.9%, 1.5%, and 0.1% for TC0, TC50/50, and TC100, respectively.

TCs were manufactured with components (cellulose acetate filters, papers, and adhesives) and construction processes consistent with commercial US cigarette manufacturing, including casing material (high fructose corn syrup) and a humectant (glycerol). The tobacco blend comprised flue-cured (63.3%), burley (25.7%), and Oriental (10.9%). The TCs were targeted to be 84 mm in length (57 mm tobacco rod, 27 mm filter) and 25 mm in circumference. Final tobacco weight was intended to be around 0.7 g. The filter material was cellulose acetate containing 8% triacetin.

Reference cigarettes, 1R4F (used by INBIFO) and 2R4F (used by IITRI) (Chen and Moldoveanu, 2003; Diana and Vaught, 1990) were used as internal assay controls. The blend composition of 2R4F was reported as 32.5% flue-cured, 19.9% burley, 1.2% Maryland, 11.1% Oriental, 27.1% reconstituted (Chen and Moldoveanu, 2003). 1R4F and 2R4F reference cigarettes were provided by the University of Kentucky Tobacco and Health Research Institute but had been previously manufactured at PM USA facilities.

Following International Organization for Standardization (ISO) standards, reference cigarettes and TCs were conditioned prior to use for 6–7 days at target conditions of 22 °C and 60% relative humidity (International Organization for Standardization, 1999). For MS chemistry endpoints, a single-port Borgwaldt/KC (Richmond, VA) smoking machine [carbon monoxide (CO), carbon dioxide, nitrogen oxides (NO_x), four aldehydes] or a 20-port Borgwaldt/KC smoking machine [total particulate matter (TPM), nicotine, water] was used. The air velocity surrounding the reference cigarettes and TCs was not monitored as specified in ISO standard 3308 (International Organization for Standardization, 2000a) and resulted in 9.1 ± 0.06 puffs for the 2R4F reference cigarette.

For cytotoxicity and mutagenicity testing of TPM, MS was generated using a 20-port (by INBIFO) and 30-port (by IITRI) Borgwaldt/KC Condor smoking machine that provided smoking conditions (35 ml puff, of 2 s duration, taken once per minute, to a butt length of 35 mm) in basic conformity with ISO 3308 (International Organization for Standardization, 2000a) and ISO 4387 (International Organization for Standardization, 2000b) (e.g., bell-shaped puff profile, restricted smoking).

2.3. Physical characteristics

Physical characteristics were measured on unequilibrated 2R4F and TCs including total cigarette length (International Organization for Standardization, 2000b), filter length, tobacco rod length, cigarette circumference (International Organization for Standardization, 1998), tipping paper length, tobacco mass, filter mass, cigarette permeability (International Organization for Standardization, 2009), ventilation (International Organization for Standardization, 2002), resistance to draw (RTD) (International Organization for Standardization, 2005), and pack oven volatiles. Lengths were measured using an electronic cigarette paper length measuring instrument (Borgwaldt KC, Richmond, VA; Onno Sokki Technology, Inc., Addison, IL), mass assessed using an analytical balance (Mettler PG203-S), and circumference determined using a non-contact, scanning laser beam cigarette test station (LaserMike, KC Automation, Richmond, VA). The details of these methods are provided in the supplementary material.

2.4. Mainstream smoke chemistry

MS was generated from the three TCs and the one reference cigarette. A total of four determinations was used for each analyte. A total of 20 cigarettes per determination was used for TPM, nicotine, water, 2-nitropropane, tobacco specific nitrosamines (TSNAs), and metals. Ten cigarettes per determination were used for phenols, polyaromatic hydrocarbons (PAHs), "volatiles", and hydrogen cyanide (HCN). Five cigarettes per determination were used for CO and for NO_x. Four cigarettes per determination were used for aromatic amines and aldehydes.

The amount of TPM collected on 92 mm glass fiber filters was determined gravimetrically. Nicotine was determined by gas chromatography (GC) with a nitrogen phosphorus detector in a 2-propanol extract from the TPM filter using isoquinoline as the internal standard (International Organization for Standardization, 2000c). Water was determined in a sample from the same 2-propanol extract by Karl Fischer titration (International Organization for Standardization, 1994). The methods used to measure specific MS classes of analytes have been previously published (Gaworski et al., 2011), and a short synopsis is available in the supplementary material.

2.5. Cytotoxicity

In vitro cytotoxicity was determined using the neutral red (NR) uptake method (Putnam et al., 2002) in accordance with INVITTOX protocols (Ungar, 1992) as previously described (Gaworski et al., 2011) and as summarized in the supplementary material. Briefly, three batches of smoke condensate were collected for each experimental cigarette (12–13 cigarettes/batch) and assayed at 8 equidistant concentrations ranging from 2 to 21 cigarettes/l for the TPM fraction and 3 to 30 cigarettes/l for the gas vapor phase (GVP) fraction. Mouse embryo fibroblast 3T3 cells were used in the cytotoxicity assay in basic conformity with established guidelines (Organisation for Economic Co-operation and Development, 1997b).

2.6. Salmonella mutagenicity

Five strains of *Salmonella typhimurium* (TA98, TA100, TA102, TA1535 and TA1537) were used by IITRI and two strains (TA98 and TA100) were used by INBIFO in the plate incorporation version of the *Salmonella* reverse mutation assay (Maron and Ames, 1983) in basic conformity with established guidelines (Organisation for Economic Co-operation and Development, 1997a). INBIFO tested strains with metabolic activation, whereas IITRI tested all strains with and without metabolic activation (S9). This test is sensitive for TPM from cigarette smoke, but the sensitivity depends on the bacterial strain and the presence or absence of S9, a cofactor-supplemented, postmitochondrial fraction, prepared from the livers of rats treated with the enzyme-inducing agent Aroclor 1254. The strains most sensitive toward TPM are TA98 and TA100, with S9 activation (Roemer et al., 2002). Two batches of MS condensate were collected for each TC and reference cigarette and assayed at three doses (by INBIFO) and four doses (by IITRI). Detailed methods have been previously reported (Gaworski et al., 2011) and are summarized in the supplementary material.

2.7. Statistical methods

Statistical comparisons were made using SigmaStat (Systat Software, Point Richmond, CA); results were considered to be statistically significant at $p < 0.05$. For the chemical analysis of MS, analysis of variance (ANOVA) was used to compare the yields of the TCs, excluding the 2R4F cigarette. If significant differences were found with the ANOVA analysis, multiple pairwise comparisons were performed using Dunnett's test. Since the original chemical analysis of MS did not include a comparison of TC50/50 to TC100, this was done *post-hoc* using an ANOVA followed by Pairwise Multiple Comparison Procedures (Holm-Sidak method using SigmaPlot software). For all chemical constituents of MS (except chromium and nickel) reported on a per mg of TPM basis, a previously calculated minimum detectable difference (MDD) for the laboratory (Oldham et al., 2012) was used to evaluate the significance of the results. The MDD was calculated using the historical assay variability of the laboratory, power $(1-\beta) = 0.8$, significance level of $\alpha = 0.05$ and sample size (n). The $1/EC_{50}$ cytotoxicity values of the TCs and reference research cigarettes were evaluated by ANOVA. Mutagenicity was assessed by the *post hoc* Dunnett's test. A result was considered positive if a slope of a TC50/50 and TC100 was statistically significantly different from that of TC0.

3. Results

3.1. Physical characteristics

Except for total RTD and ventilation, the physical properties of the three TCs were all very similar, possessing less than 5% variability (Table 1). The measured parameters demonstrated that the TCs were well-made and consistent. Measurements for reference cigarette 2R4F also indicated consistent manufacturing practices, but there were differences in cigarette length and rod density, and it contained approximately 10% more tobacco than the TCs. The mean puff counts for TC0, TC50/50, and TC100 were 10, 10.2, and 10.3, respectively, whereas the 2R4F reference cigarette resulted in 9.1 puffs per cigarette.

3.2. Smoke chemistry

Comparisons of smoke chemistry analyses were made across cigarette types for TC0, TC50/50, and TC100 on a per mg TPM basis (Table 2 and Fig. 1), per mg tar basis (supplementary material, Table S1), and per cigarette basis (supplementary material, Table S2). The results for each comparison basis are provided in sections 3.2.1, 3.2.2, and 3.2.3, respectively. Overall, for all comparisons, the inclusion of increasing levels of denic tobacco resulted in significantly lower levels of nicotine, aldehydes, phenol, styrene, and TSNAs. Increases were observed for aliphatic hydrocarbons, aliphatic nitrogen compounds, aromatic amines, vinyl chloride, NO_x, benzene, toluene, and catechol.

3.2.1. Comparisons based on TPM

On a per mg TPM basis, the denic cigarettes TC50/50 and TC100 contained lower nicotine as compared with TC0 cigarettes (Table 2, Fig. 1). Nicotine significantly decreased directionally in a dose-dependent manner (39–89%), while tar was significantly increased (4–11%) in dose-response fashion. CO was slightly increased in TC100

Table 1
Physical characteristics of research cigarettes manufactured with various levels of denicotinized tobacco filler.

Characteristic (unit)	Measurement (mean ± SD)			
	2R4F	Test cigarette		
		TC0	TC50/50	TC100
Total RTD (mm of water)	140 (5.90)	125 (5.02)	120 (6.95)	121 (6.13)
Length (mm)	84.2 (0.25)	83.9 (0.17)	83.7 (0.19)	83.8 (0.14)
Circumference (mm)	24.82 (0.05)	24.80 (0.07)	24.75 (0.04)	24.80 (0.03)
Weight (g)	1.055	0.9710 (0.002)	0.9760 (0.01)	0.9830 (0.01)
Paper permeability (CU)	25 (1.50)	34 (3.54)	35 (4.82)	34 (3.26)
Filter RTD (mm of water)	117 (5.80)	116 (3.45)	112 (3.24)	113 (3.18)
Filter tipping paper length (mm)	32.0	32.0 (0.001)	32.0 (0.001)	32.0 (0.001)
Filter ventilation (%)	27 (1.90)	27 (4.17)	29 (4.87)	29 (3.61)
Filler rod density (g/cc)	0.280	0.255	0.258	0.260
Tobacco weight (g)	0.785 (0.002)	0.709 (0.002)	0.713 (0.01)	0.721 (0.01)
Pack oven volatiles (%)	13.2 (0.080)	11.5 (0.10)	11.8 (0.13)	12.1 (0.0010)
Plug/paper weight (g)	–	0.262 (0.002)	0.264 (0.001)	0.262 (0.002)
Butt length (mm)	35.0	35.0	35.0	35.0

RTD = resistance to draw. – = not determined.

Table 2
Mainstream smoke constituents (per mg TPM) and group comparisons of smoke constituents (per mg TPM) in absolute terms and as a percentage of the value for TC0. Mean ± SD (n = 4).

Analyte	Units	2R4F	Test cigarette			Value as % of TC0	
			TC0	TC50/50	TC100	TC50/50	TC100
FTC parameters							
TPM	mg	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.03	1.00 ± 0.02	100	100
Tar	mg	0.826 ± 0.014	0.816 ± 0.023	0.849 ± 0.031	0.904 ± 0.014	104	111[a,b,c]
Nicotine	mg	0.0738 ± 0.0014	0.0984 ± 0.0026	0.0600 ± 0.0014	0.0109 ± 0.00020	61.0[a,c]	11.1[a,b,c]
Water	mg	0.0955 ± 0.011	0.0856 ± 0.0039	0.0834 ± 0.0013	0.0857 ± 0.0075	97.4	100
CO	mg	1.22 ± 0.042	0.904 ± 0.019	0.899 ± 0.020	0.945 ± 0.052	99.4	105[a,b]
Aliphatic hydrocarbons							
1,3-Butadiene	µg	4.17 ± 0.21	4.34 ± 0.27	4.31 ± 0.14	5.20 ± 0.46	99.3	120[a,b,c]
Isoprene	µg	36.5 ± 2.5	44.5 ± 2.2	44.3 ± 0.80	49.5 ± 3.3	99.6	111[a,b]
Aldehydes							
Formaldehyde	µg	1.68 ± 0.22	1.14 ± 0.088	0.787 ± 0.047	0.771 ± 0.089	69.0[a,c]	67.6[a,c]
Acetaldehyde	µg	53.7 ± 0.70	40.0 ± 0.45	41.0 ± 1.2	43.5 ± 1.5	103	109[a,b]
Acrolein	µg	5.10 ± 0.15	4.12 ± 0.15	4.04 ± 0.15	4.36 ± 0.12	98.1	106[b]
Propionaldehyde	µg	4.05 ± 0.078	3.18 ± 0.029	3.26 ± 0.071	3.40 ± 0.11	103	107[b]
Aliphatic nitrogen compounds							
Acrylonitrile	µg	1.04 ± 0.14	1.06 ± 0.10	1.12 ± 0.017	1.23 ± 0.17	106	116
HCN	µg	12.2 ± 0.71	10.6 ± 0.52	11.1 ± 0.84	13.1 ± 0.59	105	124[a,b,c]
2-Nitropropane	ng	1.23 ± 0.13	0.912 ± 0.059	0.975 ± 0.036	1.10 ± 0.10	107	121[a,c]
Acetamide	µg	0.484 ± 0.039	0.630 ± 0.030	0.650 ± 0.034	0.720 ± 0.032	103	114[a,b]
Aromatic amines							
o-Toluidine	ng	4.55 ± 0.14	4.43 ± 0.18	4.60 ± 0.068	4.73 ± 0.15	104	107[a]
2-Naphthylamine	ng	0.545 ± 0.022	0.554 ± 0.037	0.671 ± 0.013	0.749 ± 0.061	121[a,c]	135[a,b,c]
4-Aminobiphenyl	ng	0.105 ± 0.0019	0.110 ± 0.0096	0.132 ± 0.0048	0.155 ± 0.014	120[a,c]	141[a,b,c]
o-Anisidine	ng	0.249 ± 0.0054	0.207 ± 0.0088	0.220 ± 0.011	0.228 ± 0.0091	106	110[a]
Halogen compounds							
Vinyl chloride	ng	3.57 ± 0.49	1.87 ± 0.22	2.54 ± 0.24	2.75 ± 0.42	136[a,c]	147[a,c]
Inorganic compounds							
NOx	mg	0.0303 ± 0.00088	0.0202 ± 0.00070	0.0218 ± 0.00084	0.0238 ± 0.00085	108[a]	118[a,b,c]
Monocyclic aromatic compounds							
Benzene	µg	3.93 ± 0.41	3.55 ± 0.24	3.69 ± 0.030	3.94 ± 0.51	104	111
Toluene	µg	5.63 ± 0.82	5.43 ± 0.49	5.92 ± 0.26	6.68 ± 0.31	109	123[a,b,c]
Styrene	µg	0.352 ± 0.065	0.437 ± 0.015	0.433 ± 0.040	0.413 ± 0.087	99.1	94.5
N-nitrosamines							
NDMA	ng	0.343 ± 0.044	0.254 ± 0.038	0.188 ± 0.031	0.246 ± 0.026	74.0	96.9
NMEA	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NDEA	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NPRA	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NBUA	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NPY	ng	0.546 ± 0.082	0.448 ± 0.075	0.433 ± 0.059	0.505 ± 0.037	96.7	113
NPI	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
NNN	ng	12.7 ± 0.33	10.5 ± 0.59	7.56 ± 0.29	4.86 ± 0.16	72.0[a,c]	46.3[a,b,c]
NNK	ng	13.8 ± 0.30	7.96 ± 0.28	6.03 ± 0.34	4.07 ± 0.40	75.8[a,c]	51.1[a,b,c]
Phenols							
Phenol	µg	0.901 ± 0.085	1.39 ± 0.12	1.06 ± 0.069	0.843 ± 0.031	76.3[a,c]	60.6[a,b,c]
Catechol	µg	3.70 ± 0.20	3.95 ± 0.10	4.14 ± 0.12	4.54 ± 0.14	105	115[a,b,c]
Polycyclic aromatic hydrocarbons							
Benz[*a*]anthracene	ng	1.17 ± 0.062	0.960 ± 0.077	0.966 ± 0.11	1.11 ± 0.065	101	116[c]
Benzo[*b*]fluoranthene	ng	0.495 ± 0.020	0.425 ± 0.018	0.441 ± 0.013	0.445 ± 0.011	104	105
Benzo[*k*]fluoranthene	ng	0.213 ± 0.014	0.191 ± 0.014	0.192 ± 0.013	0.195 ± 0.0071	101	102
Benzo[*j*]fluoranthene	ng	0.324 ± 0.0064	0.281 ± 0.0080	0.292 ± 0.0078	0.289 ± 0.020	104	103
Benzo[*a*]pyrene	ng	0.671 ± 0.019	0.587 ± 0.015	0.606 ± 0.018	0.603 ± 0.014	103	103
Indeno[1,2,3-*cd*]pyrene	ng	0.304 ± 0.0068	0.265 ± 0.010	0.270 ± 0.0084	0.268 ± 0.012	102	101
Dibenz[*a,h*]anthracene	ng	0.025 ± 0.0020	0.019 ± 0.0028	0.021 ± 0.0018	0.018 ± 0.0024	111	94.7
5-Methylchrysene	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenzo[*a,l*]pyrene	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenzo[*a,e*]pyrene	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenzo[*a,i*]pyrene	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenzo[*a,h*]pyrene	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Metals							
Arsenic	ng	0.324 ± 0.0066	0.424 ± 0.015	0.467 ± 0.0084	0.517 ± 0.0091	110[a]	122[a,b,c]
Cadmium	ng	5.40 ± 0.10	5.94 ± 0.21	6.26 ± 0.21	6.68 ± 0.24	105	112[a,b,c]
Chromium	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Nickel	ng	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Lead	ng	1.29 ± 0.056	0.872 ± 0.038	0.924 ± 0.072	0.909 ± 0.019	106	104

CO = carbon monoxide, FTC = Federal Trade Commission, HCN = hydrogen cyanide, n.d. = none detected (below the level of quantification), NBUA = *N*-nitrosodi-n-butylamine, NDEA = *N*-nitrosodiethylamine, NDMA = *N*-nitrosodimethylamine, NMEA = *N*-nitrosomethylethylamine, NNN = *N*-nitrosonornicotine, NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, NO_x = nitrogen oxides, NPI = *N*-nitrosopiperidine, NPRA = *N*-nitroso-*N*-propylamine, NPY = *N*-nitrosopyrrolidine, TPM = total particulate matter.

[a]Mean values are statistically significantly different ($p < 0.05$) from the mean value for the TC0 group (excluding 2R4F).

[b]Mean values are statistically significantly different ($p < 0.05$) from the mean value for the TC50/50 group (excluding 2R4F).

[c]Difference between TC0 and either TC50/50 or TC100 exceeded the minimum detectable difference (MDD) using the historical assay variability of the laboratory, power (1-β) = 0.8, significance level of α = 0.05 and sample size (n). MDD for chromium and nickel were not available.

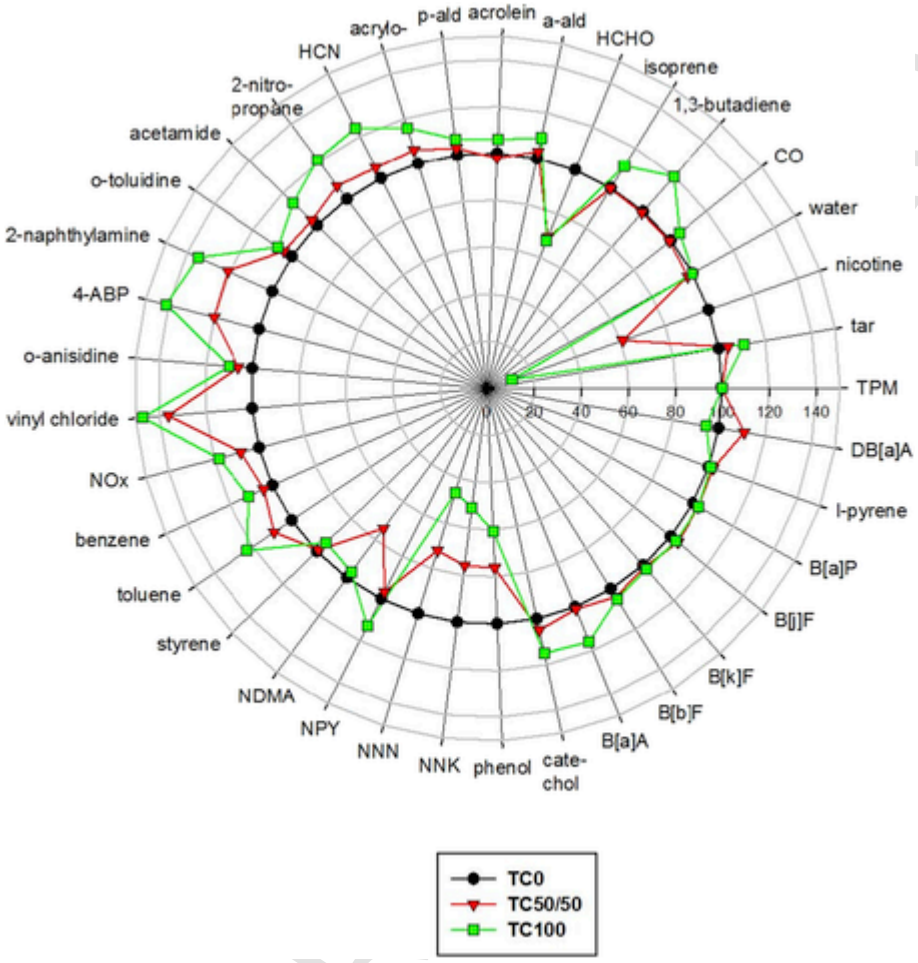

Fig. 1. Radar plot for % changes in analyte concentrations (compared to TC0) of test cigarettes with the inclusion of various levels of denicotinized tobacco (per mg TPM basis).

but did not exceed the MDD. Although aliphatic hydrocarbons were increased for TC50/50 and TC100, only 1,3-butadiene in TC100 exceeded the MDD. Formaldehyde was significantly reduced (31–33%) for both TC50/50 and TC100, whereas changes in acetaldehyde and propionaldehyde did not exceed the MDD for either TC50/50 or TC100. Aliphatic nitrogen compounds displayed dose-related increases (3–24%) for both TC50/50 and TC100; however, only in TC100 did HCN and 2-nitropropane exceed the MDD. Aromatic amines were generally increased in a dose-related fashion for all analytes, with 2-naphthylamine and 4-aminobiphenyl in TC100 exceeding the MDD. Vinyl chloride was significantly increased (36–47%) and exceeded the MDD for both TC50/50 and TC100, while NO_x increases (8–18%) exceeded the MDD for only TC100 in a dose-related fashion. With the exception of styrene, monocyclic aromatic compounds were generally increased (4–23%) in a dose-related trend from TC0, and toluene exceeded the MDD in TC100. TSNAs were generally decreased for one or both of the TCs while *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were statistically significantly lower and exceeded the MDD although not in a dose-related fashion, and many TSNAs were below the limits of detection. Phenol was significantly decreased (~23–40%) and exceeded the MDD for both TC50/50 and TC100 while catechol was significantly increased (5–15%) and exceeded the MDD in TC100. PAHs were only slightly elevated with the exception of; benz[*a*]anthracene, which was statistically significantly elevated by 16% (exceeded the MDD) in TC100, and dibenz[*a,h*]anthracene was elevated by 11% in TC50/50 and approximately 5.3% lower in TC100 compared with TC0 (neither exceeded the MDD).

Arsenic was significantly increased in MS condensate of TC50/50 and TC100 as compared with TC0 (Table 2) and exceeded the MDD. Cadmium was significantly elevated in TC100 as compared with TC0 and exceeded the MDD. There were no other consistent changes for the other metals in smoke that appeared to be correlated to the composition of the TCs.

3.2.2. Comparisons based on tar

On a per mg tar basis of comparison (supplementary material, Table S1), TPM, nicotine, water, and CO were all reduced in TC50/50 and TC100. Aldehydes were generally slightly lower, while formaldehyde was significantly lower in MS condensate of TC50/50 and TC100. Aliphatic hydrocarbons were unchanged and aliphatic nitrogen compounds were slightly increased. 2-Napthylamine and 4-aminobiphenyl were significantly increased in a dose-related fashion, while the other aromatic amines were unchanged. We observed slight elevations in NO_x levels, significant increases in vinyl chloride, elevations in toluene with no other changes in monocyclic aromatic compounds. We also observed dose-related significant reductions in TSNAs (NNN, NNK) and general decreases in PAHs with significant reductions in benzo[*a*]pyrene and indeno[1,2,3-*cd*]pyrene in MS condensate of TC50/50 and TC100. Comparing TC50/50 and TC100, significant reductions were observed for TPM, nicotine, NNN, NNK, phenol, and benzo[*a*]pyrene at the higher denic tobacco inclusion level (TC100).

Arsenic was significantly increased in MS condensate of TC50/50 and TC100, but did not exceed the MDD (supplementary material, Table S2).

3.2.3. Comparisons by cigarette

On a per cigarette basis (supplementary material, Table S2), similar trends were observed. Nicotine was significantly reduced by 42% and 90%, respectively, for TC50/50 and TC100. TPM was significantly reduced approximately 5% and 12% for TC50/50 and TC100, respectively.

Aliphatic hydrocarbons were generally unchanged, while aldehydes generally had small but statistically significant reductions except for formaldehyde, which was reduced by 34–40%. Aliphatic nitrogen compounds were nearly unchanged. Select aromatic amines and vinyl chloride were significantly increased for TC50/50 and TC100. NO_x and monocyclic aromatics were unchanged on a per cigarette basis. TSNAs were generally statistically significantly lower, phenol was reduced, catechol was unchanged, and PAHs were generally statistically significantly lower. The inclusion of more denic tobacco from TC50/50 to TC100 resulted in statistically significant decreases in TPM and nicotine and statistically significant decreases in NNN, NNK, and all the PAHs above the limit of quantification except benz[*a*]anthracene, which was increased.

Arsenic was significantly increased in MS condensate of TC100, but did not exceed the MDD (supplementary material, Table S2).

3.3. Cytotoxicity

There were no statistically significant differences in cytotoxicity among TC0, TC50/50, and TC100 in either the GVP or particulate fraction of the MS on a per mg TPM (Table 3). There were also no significant differences in cytotoxicity between TC0, TC50/50, and TC100 in GVP or particulate fraction of MS when expressed on a per mg tar or per cigarette basis (supplementary material, Table S3).

On a per mg TPM basis, the particulate fraction of MS from the 2R4F reference cigarette was statistically significantly less cytotoxic than that from TC0, and it was similarly less cytotoxic than TC0 on a per mg tar basis, although not statistically significant. However, the cytotoxicity of GVP from TC0 and 2R4F cigarettes was similar on a per mg tar and per mg TPM basis.

Conversely, the cytotoxicity of the particulate fraction of TC100 was the highest on a per mg TPM and per mg tar basis, without being statistically significant. Furthermore, it appeared that the particulate phase of MS produces a greater cytotoxic response (compared with GVP) whether the cigarette is a "conventional" product (e.g., 2R4F, TC0) or denic to varying degrees (e.g., TC50/50 or TC100).

Table 3
Cytotoxicity, expressed as $1/EC_{50}$, in absolute terms and as a percentage of the value for TC0.

Parameter and laboratory	Run #	$1/EC_{50}$ TPM ($1/EC_{50}$ GVP)					
		2R4F[a]	Test cigarette			Value as % of TC0	
			TC0	TC50/50	TC100	TC50/50	TC100
Per mg TPM							
IITRI	Mean	5.08 (4.59)	6.84 (3.67)	6.56 (3.62)	7.76 (3.82)	96 (99)	113 (104)
	Range (+/−)[b]	0.30 (0.18)	0.030 (0.40)	0.61 (0.25)	0.060 (0.08)		
INBIFO	Mean	–	8.80 (5.15)	8.28 (5.25)	9.74 (5.53)	94 (102)	111 (107)
	SD	–	1.45 (0.27)	0.930 (0.36)	0.190 (0.41)		

INBIFO = Philip Morris Research Laboratory, previously called Institut fur Biologische Forschung; IITRI = IIT Research Institute. GVP = gas vapor phase; TPM = total particulate matter. – = not determined.

[a] No data for 2R4F reference cigarette at INBIFO as this cigarette was unavailable during the study period.

[b] Plus/minus (range) provided because only two runs were performed.

3.4. Salmonella mutagenicity

Comparisons of the mutagenic responses in *Salmonella* strains TA98, TA100, TA102, TA1535, and TA1537 with the S9 fraction showed no statistically significant differences for MS condensate from TC0, TC50/50, and TC100 experimental cigarettes on per mg TPM basis (Table 4). No mutagenic activity was seen in any *Salmonella* tester strain when the S9 fraction was not used (Table 4). Mutagenic responses for the 2R4F reference cigarette were within normal ranges. Comparison of the mutagenic responses on a per mg tar and per cigarette basis (supplementary material, Tables S4 and S5, respectively) in *Salmonella* strains TA98, TA100, TA102, TA1535, and TA1537 with and without S9 activation were similar to those on a per mg TPM basis.

4. Discussion

Several reduced nicotine cigarettes (Quest®, PM USA denic RCs, and now recently SPECTRUM®) have been used in numerous clinical studies assessing the effects of VLN cigarettes on smoking cessation and nicotine dependence (Donny et al., 2015; Bandiera et al., 2014; Dermody et al., 2014; Walker et al., 2014; Cummins, 2014; Hatsukami et al., 2013; Benowitz et al., 2012). However, little has been published regarding the construction and toxicological effect of these RCs, with the exception of Quest® (Chen et al., 2008; Strasser et al., 2006) A comparison of Quest® and SPECTRUM® RCs, which use genetically modified (GMO) tobacco results in reduced nicotine levels similar to that of PM USA RCs, which are made with tobacco that is denicotinized using a supercritical CO_2 extraction method. Here, we have reported on the physical characteristics, smoke chemistry, and *in vitro* cytotoxicity and mutagenicity of PM USA TCs manufactured with 0%, 50%, and 100% inclusion of denic tobacco filler. We found significant differences in select smoke constituents,

Table 4
Revertant *Salmonella* colonies per mg TPM, in absolute values and as a percentage of the value for TC0.

Laboratory, strain (activation)	Parameter	2R4F[a]	Test cigarette			Value as % of TC0	
			TC0	TC50/50	TC100	TC50/50	TC100
INBIFO							
TA98 (+S9)	Mean	–	3944	4218	3952	107	100
	Range (±)	–	184	245	388		
TA100 (+S9)	Mean	–	1321	1531	1452	116	110
	Range (±)	–	108.5	68.50	79.00		
IITRI							
TA98 (-S9)	Mean	7.0	14	11	5.0	78	36
	SD	2.1	2.8	7.1	4.2		
TA100 (-S9)	Mean	123	45.0	81.0	19.0	180	42
	SD	55.2	12.7	70.7	36.1		
TA102 (-S9)	Mean	28	27	7.0	−31	26	–
	SD	29.7	26.9	50.9	74.2		
TA1535 (-S9)	Mean	3	5	2	1	20	40
	SD	2.8	2.1	0.70	2.1		
TA1537 (-S9)	Mean	5	3	8	3	267	100
	SD	1.4	4.2	4.9	4.9		
TA98 (+S9)	Mean	1549	1842	2134	2302	116	125
	SD	175	454	125	55.0		
TA100 (+S9)	Mean	251	265	348	393	131	148
	SD	95.0	108	99.0	175		
TA102 (+S9)	Mean	55	48	21	88	44	183
	SD	4.0	35	38	23		
TA1535 (+S9)	Mean	1	2	−1	1	–	50
	SD	3.5	0.70	0.00	1.0		
TA1537 (+S9)	Mean	171	230	247	207	107	90
	SD	14	51	37	64		

INBIFO = Philip Morris Research Laboratory, previously called Institut fur Biologische Forschung; IITRI = IIT Research Institute. – = not determined.

[a] No data for 2R4F reference cigarette at INBIFO as this cigarette was unavailable during the study period.

but overall no alterations in the *in vitro* toxicological effect of denic TCs compared with control unextracted TCs, as demonstrated by NR uptake in mammalian cells and bacterial mutagenicity testing.

4.1. Physical characteristics

The physical characteristics of the three TCs demonstrate they were reproducibly constructed and permit a direct comparison of MS chemistry, *in vitro* cytotoxicity and *Salmonella* mutagenicity. In contrast, the difference in tobacco filler weight, RTD, and filler rod density resulted in puff count differences between the three TCs and the 2R4F reference cigarettes. These differences between TCs and the 2R4F reference cigarettes indicate that normalization per amount of TPM or tar is necessary for a valid comparison of smoke chemistry and *in vitro* toxicity. There is limited published information on the physical characteristics of VLN RCs, with the exception of Quest® RCs (Donny et al., 2015; Strasser et al., 2006). An assessment of physical attributes between Quest® cigarettes and PM USA RCs illustrates a significant difference in their cigarette design (Strasser et al., 2006). Unlike PM USA RCs, Quest® cigarettes contain activated charcoal (0.1 g/cigarette) in the filter, are devoid of filter ventilation, are lower in puff count (6.4 puffs/cigarette, by Federal Trade Commission method), and contain 20% less tobacco mass/cigarette, all of which could potentially alter MS chemistry (Strasser et al., 2006). The presence of activated charcoal in the filter has been shown to significantly reduce the yields of GVP constituents such as monocyclic aromatic compounds, aldehydes, aliphatic hydrocarbons, aliphatic nitrogen compounds, and cadmium (Gaworski et al., 2009). Recently limited physical design parameters have been published for SPECTRUM® RCs, of which include slightly less tobacco per cigarette (0.68 g) and similar moisture levels (12.9%) as compared to PM USA RCs (Donny et al., 2015). Therefore, to enable comparisons of smoke chemistry and *in vitro* toxicity results between different VLN tobacco RCs, it is essential that physical characteristics be reported. The full physical characteristic profile of SPECTRUM RCs is not known.

4.2. Smoke chemistry

In the present study, we report the smoke chemistry for 47 analytes, 43 of which are included in the list of harmful and potentially harmful constituents (HPHCs) published by US Food and Drug Administration (FDA) in 2012 (US Food and Drug Administration Center for Tobacco Products, 2012). The selection of these 47 analytes was made prior to the publication of the HPHC list and was based on the scientific literature (Gaworski et al., 2011) and our accumulated experience studying the toxicology of cigarette smoke. In addition, the biologic assessment of whole smoke or smoke fractions would likely capture the effects of smoke constituents not measured. Smoke constituent data can be presented in various ways using several calculation bases, such as on a per mg TPM, per mg tar, or per cigarette basis. Here we report smoke constituent data expressed on a "per mg TPM" (Table 2), "per mg tar" (supplementary material, Table S1) and "per cigarette" basis (supplementary material, Table S2).

We observed significant changes in the levels of smoke constituents, including decreases in formaldehyde, nitrosamines, and phenol and increases in aliphatic hydrocarbons, aliphatic nitrogen compounds, aromatic amines, halogen compounds, and metals. The FDA list of HPHCs identifies and classifies constituents as being carcinogens, respiratory toxicants, cardiovascular toxicants, reproductive and developmental toxicants and as addictive (US Food and Drug Administration Center for Tobacco Products, 2012). The specific role of these toxicants in cigarette smoke-mediated disease is unclear. However, the toxicological hazard classification of these constituents has been reported in the scientific literature (Burns et al., 2008; Life Sciences Research Office, 2004; Shields, 2002; Thun et al., 2002). The decreasing concentrations (up to 50%) of the TSNAs NNN and NNK observed (see Fig. 1) is presumably directly related to the reduction of nicotine and possibly other tobacco alkaloids occurring during the supercritical CO_2 fluid extraction process (Morgan, 2000). A number of investigators have shown that nicotine and other nicotine-related alkaloids may serve as precursors in the complex nitrosation chemistry of TSNA formation during curing and burning of a

tobacco cigarette (Adam et al., 2010). Reducing the nicotine precursor lowers one or possibly more amine-containing constituents in the chemical reaction process, thereby reducing formation of the TSNA product (Adam et al., 2010). Chen et al. (2008) reported a similar decrease in NNK values between Quest® 1 (low nicotine GMO tobacco) and Quest® 3 (VLN GMO tobacco) cigarettes, but interestingly, they observed the opposite effect for NNN values. An explanation for this difference is currently unknown. Unlike Quest® cigarettes, a significant reduction in the MS levels of both NNN and NNK was reported for SPECTRUM® VLN (0.4 mg/g nicotine) RCs as compared to control (15.8 mg/g nicotine RCs), as characterized by the Center for Disease Control and Prevention (Donny et al., 2015).

Chen et al. (2008) also reported that smoke constituent values (per cigarette) from Quest® cigarettes were reduced for most analytes as compared with 2R4F reference cigarettes. However, interpretation of such a comparison is difficult since Quest® cigarettes contained 30% less tobacco mass/cigarette and contained activated charcoal. On a cigarette basis of comparison, it is plausible that a 30% decrease in tobacco mass could result in significant reductions in TPM and tar yield. The gas phase components of the smoke yield may have been further reduced by the presence of activated carbon in the filter of Quest cigarettes, which is used to scavenge volatile organic hydrocarbons from smoke GVP (Gaworski et al., 2009). Both of these physical attributes would obviously lead to a decrease in smoke analyte levels. However, despite these differences in physical characteristics, a significant increase in aromatic amines was observed in MS from Quest® 3 cigarettes as compared with 2R4F (similar to that observed with denic tobacco TCs). Therefore, the observed differences in the smoke chemistry profiles of Quest® and PM USA denic RCs could be attributed to both dissimilarities in cigarette design and the processes used to reduce the nicotine content. The full smoke chemistry profile of SPECTRUM® RCs is not known.

4.3. In vitro cytotoxicity and mutagenicity

Cigarette smoke has been repeatedly shown to be cytotoxic and mutagenic (DeMarini et al., 2008; Patskan et al., 2008; Rickert et al., 2007; Bombick et al., 1997). Similar assay systems were used in the present study to measure cytotoxicity (mouse embryo fibroblast 3T3 cells) and mutagenicity (*Salmonella typhimurium* reverse mutation assay) and are in basic conformity with OECD guideline no. 432 and no. 471 respectively (Organisation for Economic Co-operation and Development, 1997b; Organisation for Economic Co-operation and Development, 1997a). These cytotoxicity and mutagenicity assays have been routinely used to evaluate potential adverse effects of ingredients added to cigarettes and "were selected based on their known sensitivity to cigarette smoke and their recognized predictivity for potential biological activity" (Carmines, 2002; Roemer et al., 2002). Though most ingredient testing studies report statistically significant changes in various constituents of cigarette smoke, no significant increases in biological activity were observed using these assay systems (Roemer et al., 2002, 2014). In contrast, cigarettes prepared with different tobacco types or cigarettes with tobacco electrically heated were found to have significant changes in smoke constituents with a statistically significant increase or decrease in cytotoxicity respectively using the 3T3/neutral red uptake cytotoxicity assay (Roemer et al., 2004). In the present study, use of the supercritical CO_2 extraction method for removal of nicotine from the tobacco did not significantly influence *in vitro* cytotoxic or mutagenic activity of MS. While the *in vitro* responses were generally not statistically significant, small changes emerged suggesting a potential impact of some smoke constituent differences for the TC100 blend compared to the TC0 and TC50/50 blends. For example, the measured smoke aldehydes were slightly increased on the order of 6–9%, and formaldehyde was lower by nearly 30% while the corresponding cytotoxicity responses for TC100 tobacco were increased slightly, the prevailing response being a very small non-statistically significant increase in cytotoxicity. For the Ames test no consistent pattern of changes was evident in any strain with or without the S9 fraction, possibly due to complex interactions between some known mutagens in smoke that increased (aromatic amines) some very slightly (PAHs) while others decreased significantly (NNN and NNK). The overall conclusion from these studies is that the denicotinization of tobacco using a supercritical CO_2 extraction method resulted in a VLN tobacco RC that did not exhibit a change in *in vitro* cytotoxicity or mutagenicity as compared to unextracted tobacco RC or reference RC.

PM USA denic tobacco RCs, like Quest® cigarettes, when compared to nicotine-containing RC were found to be of similar cytotoxic and mutagenic potential, despite possessing dissimilar smoke chemistry profiles (Chen et al., 2008). The *in vitro* toxicological profile of SPECTRUM® RCs is unknown. The one smoke analyte that was found to be dramatically reduced by approximately 90–97% (compared with reference RCs) in all three types of VLN tobacco RCs was nicotine, which of course was by design. These findings appear to suggest that a dramatic reduction in smoke nicotine content has little influence on the cytotoxic or mutagenic potential of MS. This is somewhat surprising since previous *in vitro* studies investigating nicotine per se have reported cytoprotective and mutagenic activity (Haussmann and Fariss, 2016; Grando, 2014). These observed effects of nicotine are controversial, as numerous conflicting studies have been reported (Haussmann and Fariss, 2016). It is well established that nicotine's biological effects are predominately mediated by binding and activating nicotinic acetylcholine receptors (nAChRs) in a wide variety of neuronal and non-neuronal tissue. The presence of different types of nAChRs, receptor up regulation and receptor desensitization influence these complex physiological effects (Lam et al., 2016; Brown et al., 2013; Improgo et al., 2013; Marks et al., 1985). Many types of cancer cells express a wide variety of nAChRs (Improgo et al., 2013). As a result, the vast majority of *in vitro* studies investigating the cytoprotective potential of nicotine per se were conducted with cancer cell lines exposed to a variety of anti-tumor treatments (Grando, 2014; Banerjee et al., 2013; Cardinale et al., 2012; Warren et al., 2012; Maneckjee and Minna, 1994). For example, apoptotic cell death in human lung cancer cells exposed to opioids (Maneckjee and Minna, 1994), cisplatin (Cardinale et al., 2012) or radiation (Warren et al., 2012) was suppressed by nicotine at levels observed with tobacco use.

It is known that mouse embryo fibroblast 3T3 cells do not express active nAChRs (Aztiria et al., 2000) and therefore we speculate that this cell type would be relatively unaffected by nicotine at levels found in MS. Therefore, it is a challenge to predict the biological effect of dramatically reducing nicotine levels from tobacco using mammalian cells that do not express active nAChRs. As normal lung cells and tissue have been reported to express active nAChRs (Lam et al., 2016; Fu et al., 2009), it would be interesting to determine the cytoprotective effect of nicotine against toxicants such as cigarette smoke condensate in human normal lung cells expressing nAChRs. Such an assay system might prove useful in future studies assessing the potential hazardous effect of VLN tobacco cigarettes.

4.4. Other studies

A published study indicates increased toxicity among blood brain barrier endothelial cells exposed to whole smoke extract from VLN (0.03 mg) SPECTRUM® RCs cigarettes as compared with 1R5F and

3R4F reference RCs and commercially available Marlboro® cigarettes (Naik et al., 2014). In addition, the researchers reported increased nitrate and nitrite concentrations in SPECTRUM® (0.03 mg nicotine) cigarettes (Naik et al., 2014). On the other hand, atherosclerotic lesions of the aorta were observed to be significantly smaller in Apo $E^{-/-}$ mice exposed to cigarette smoke from Quest® 3 cigarettes (0.05 mg nicotine) as compared with Quest® 1 (0.6 mg nicotine) or 2R4F reference cigarettes (Catanzaro et al., 2007). These reports suggest additional studies are required for a better understanding of the toxicological potential of VLN tobacco cigarettes.

4.5. Limitations

The supercritical CO_2 extraction process does not selectively remove solely nicotine, but also results in the elimination of other tobacco alkaloids and plant waxes (Morgan, 2000). It is well known that carbohydrate-containing tobacco leaf polymers, lipids, and waxes are responsible, in part, for formaldehyde yields in cigarette smoke (Piade et al., 2013). It is interesting to note that formaldehyde yields were dramatically reduced using denic tobacco cigarettes (Table 2, Fig. 1). Therefore, non-nicotine associated changes in the tobacco resultant from the extraction procedure, may influence the toxicological profile of RCs containing denic tobacco. In addition, the RCs used in the studies conducted by INBIFO and IITRI were manufactured using the same process, but at different times. To account for manufacturing batch differences, reference cigarettes such as 1R4F and 2R4F were included as internal assay controls and were compared with historical data. Here, we provide data from analytical and *in vitro* toxicology studies conducted in the early 2000s on TCs manufactured with various amounts of denic tobacco.

5. Conclusions

Removal of nicotine from the tobacco via use of a supercritical CO_2 extraction process resulted in several alterations in the chemical composition of MS, but the changes did not modify biological activity as measured by the cytotoxicity and mutagenicity assays used. The physical characteristics of the TCs were very reproducible and similar to 2R4F, but differed from that of Quest® 3 cigarettes. Unlike PM USA denic RCs, Quest® 3 cigarettes possess ventilation, 20% more tobacco mass/cigarette, and they contain activated carbon in the filter. As more data becomes available it will be interesting to see how the physical and chemical profiles of SPECTRUM® RCs compare with those of other genetically modified VLN RCs (Quest® 3) and nicotine-extracted RCs such as PM USA denic RCs.

Funding

This work was supported by Philip Morris USA Inc. and Altria Client Services Inc.

Declaration of interests

Dr. C.R.E. Coggins is a consultant for Altria Client Services Inc. and as such was compensated for his contribution to this study.

Acknowledgments

The authors acknowledge the study directors at Philip Morris USA and Philip Morris Research Laboratories, IIT Research Institute, as well as Mohamadi Sarkar. The authors also acknowledge the editorial assistance of Eileen Y. Ivasauskas of Accuwrit Inc.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.yrtph.2016.05.016.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2016.05.016.

References

Adam, T., McAughey, J., Mocker, C., McGrath, C., Zimmermann, R., 2010. Influence of filter ventilation on the chemical composition of cigarette mainstream smoke. Anal. Chim. Acta 657, 36–44.

Aztiria, E.M., Sogayar, M.C., Barrantes, F.J., 2000. Expression of a neuronal nicotinic acetylcholine receptor in insect and mammalian host cell systems. Neurochem. Res. 25, 171–180.

Bandiera, F.C., Arguelles, W., Gellman, M., Castaneda, S.F., Barnhart, J., Gonzalez, P., Navas-Nacher, E.L., Salgado, H., Talavera, G.A., Schneiderman, N., Lee, D.J., 2014. Cigarette smoking and depressive symptoms among Hispanic/Latino adults: results from the Hispanic community health study/study of Latinos (HCHS/SOL). Nicotine Tob. Res. http://dx.doi.org/10.1093/ntr/ntu209.

Banerjee, J., Al-Wadei, H.A., Schuller, H.M., 2013. Chronic nicotine inhibits the therapeutic effects of gemcitabine on pancreatic cancer in vitro and in mouse xenografts. Eur. J. Cancer 49, 1152–1158.

Benowitz, N.L., Hall, S.M., Stewart, S., Wilson, M., Dempsey, D., Jacob III, P., 2007. Nicotine and carcinogen exposure with smoking of progressively reduced nicotine content cigarette. Cancer Epidemiol. Biomark. Prev. 16, 2479–2485.

Benowitz, N.L., Dains, K.M., Hall, S.M., Stewart, S., Wilson, M., Dempsey, D., Jacob III, P., 2012. Smoking behavior and exposure to tobacco toxicants during 6 months of smoking progressively reduced nicotine content cigarettes. Cancer Epidemiol. Biomark. Prev. 21, 761–769.

Bodnar, J.A., Morgan, W.T., Murphy, P.A., Ogden, M.W., 2012. Mainstream smoke chemistry analysis of samples from the 2009 US cigarette market. Regul. Toxicol. Pharmacol. 64, 35–42.

Bombick, D.W., Ayres, P.H., Doolittle, D.J., 1997. Cytotoxicity assessment of whole smoke and vapor phase of mainstream and sidestream cigarette smoke from three Kentucky reference cigarettes. Toxicol. Mech. Methods 7, 177–190.

Brown, K.C., Perry, H.E., Lau, J.K., Jones, D.V., Pulliam, J.F., Thornhill, B.A., Crabtree, C.M., Luo, H., Chen, Y.C., Dasgupta, P., 2013. Nicotine induces the up-regulation of the alpha7-nicotinic receptor (alpha7-nAChR) in human squamous cell lung cancer cells via the Sp1/GATA protein pathway. J. Biol. Chem. 288, 33049–33059.

Burns, D.M., Dybing, E., Gray, N., Hecht, S., Anderson, C., Sanner, T., O'Connor, R., Djordjevic, M., Dresler, C., Hainaut, P., Jarvis, M., Opperhuizen, A., Straif, K., 2008. Mandated lowering of toxicants in cigarette smoke: a description of the World Health Organization TobReg proposal. Tob. Control 17, 132–141.

Cardinale, A., Nastrucci, C., Cesario, A., Russo, P., 2012. Nicotine: specific role in angiogenesis, proliferation and apoptosis. Crit. Rev. Toxicol. 42, 68–89.

Carmines, E.L., 2002. Evaluation of the potential effects of ingredients added to cigarettes. Part 1: cigarette design, testing approach, and review of results. Food Chem. Toxicol. 40, 77–91.

Catanzaro, D.F., Zhou, Y., Chen, R., Yu, F., Catanzaro, S.E., De Lorenzo, M.S., Subbaramaiah, K., Zhou, X.K., Pratico, D., Dannenberg, A.J., Weksler, B.B., 2007. Potentially reduced exposure cigarettes accelerate atherosclerosis: evidence for the role of nicotine. Cardiovasc. Toxicol. 7, 192–201.

Chen, J., Higby, R., Tian, D., Tan, D., Johnson, M.D., Xiao, Y., Kellar, K.J., Feng, S., Shields, P.G., 2008. Toxicological analysis of low-nicotine and nicotine-free cigarettes. Toxicology 249, 194–203.

Chen, P.X., Moldoveanu, S.C., 2003. Mainstream smoke chemical analyses for 2R4F Kentucky reference cigarette. Beitr. Tabakforsch. Int. 20, 448–458.

Counts, M.E., Hsu, F.S., Tewes, F.J., 2006. Development of a commercial cigarette "market map" comparison methodology for evaluating new or non-conventional cigarettes. Regul. Toxicol. Pharmacol. 46, 225–242.

Cummins, S., 2014. Adding very low nicotine content cigarettes to nicotine replacement therapy and behavioural support increases abstinence at 6 months after the quit date. Evid. Based Nurs. 17, 19–20.

DeMarini, D.M., Gudi, R., Szkudlinska, A., Rao, M., Recio, L., Kehl, M., Kirby, P.E., Polzin, G., Richter, P.A., 2008. Genotoxicity of 10 cigarette smoke condensates in four test systems: comparisons between assays and condensates. Mut. Res. 650, 15–29.

Dermody, S.S., Donny, E.C., Hertsgaard, L.A., Hatsukami, D.K., 2014. Greater reductions in nicotine exposure while smoking very low nicotine content cigarettes predict smoking cessation. Tob. Control http://dx.doi.org/10.1136/tobaccocontrol-2014–051797.

Diana, J.N., Vaught, A., 1990. Research Cigarettes. University of Kentucky Printing Services, Lexington, KY.

Domino, E.F., Ni, L., Domino, J.S., Yang, W., Evans, C., Guthrie, S., Wang, H., Koeppe, R.A., Zubieta, J.K., 2013. Denicotinized versus average nicotine tobacco cigarette smoking differentially releases striatal dopamine. Nicotine Tob. Res. 15, 11–21.

Donny, E.C., Denlinger, R.L., Tidey, J.W., Koopmeiners, J.S., Benowitz, N.L., Vandrey, R.G., al'Absi, M., Carmella, S.G., Cinciripini, P.M., Dermody, S.S., Drobes, D.J., Hecht, S.S., Jensen, J., Lane, T., Le, C.T., McClernon, F.J., Montoya, I.D., Murphy, S.E., Robinson, J.D., Stitzer, M.L., Strasser, A.A., Tindle, H., Hatsukami, D.K., 2015. Randomized trial of reduced-nicotine standards for cigarettes. N. Engl. J. Med. 373, 1340–1349.

Fu, X.W., Lindstrom, J., Spindel, E.R., 2009. Nicotine activates and up-regulates nicotinic acetylcholine receptors in bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. 41, 93–99.

Gaworski, C.L., Schramke, H., Diekmann, J., Meisgen, T.J., Tewes, F.J., Veltel, D.J., Vanscheeuwijck, P.M., Rajendran, N., Muzzio, M., Haussmann, H.J., 2009. Effect of filtration by activated charcoal on the toxicological activity of cigarette mainstream smoke from experimental cigarettes. Inhal. Toxicol. 21, 688–704.

Gaworski, C.L., Oldham, M.J., Wagner, K.A., Coggins, C.R., Patskan, G.J., 2011. An evaluation of the toxicity of 95 ingredients added individually to experimental cigarettes: approach and methods. Inhal. Toxicol. 23 (Suppl. 1), 1–12.

Grando, S.A., 2014. Connections of nicotine to cancer. Nat. Rev. Cancer 14, 419–429.

Hatsukami, D.K., Kotlyar, M., Hertsgaard, L.A., Zhang, Y., Carmella, S.G., Jensen, J.A., Allen, S.S., Shields, P.G., Murphy, S.E., Stepanov, I., Hecht, S.S., 2010. Reduced nicotine content cigarettes: effects on toxicant exposure, dependence and cessation. Addiction 105, 343–355.

Hatsukami, D.K., Benowitz, N.L., Donny, E., Henningfield, J., Zeller, M., 2013. Nicotine reduction: strategic research plan. Nicotine Tob. Res. 15, 1003–1013.

Haussmann, H.-J., Fariss, M.W., 2016. Comprehensive review of epidemiological and animal studies on the potential carcinogenic effects of nicotine per se. Crit. Rev. Toxicol. (in press).

Improgo, M.R., Soll, L.G., Tapper, A.R., Gardner, P.D., 2013. Nicotinic acetylcholine receptors mediate lung cancer growth. Front. Physiol. 4, 251.

International Organization for Standardization, 1994. ISO 10362–2. Cigarettes – Determination of Water in Smoke Condensates – Part 2. Karl Fischer Method. International Organization for Standardization, Geneva.

International Organization for Standardization, 1998. ISO 2971. Cigarettes and Filter Rods – Determination of Nominal Diameter – Methods Using a Laser Beam Measuring Apparatus. International Organization for Standardization, Geneva.

International Organization for Standardization, 1999. ISO 3402. Tobacco and Tobacco Products – Atmosphere for Conditioning and Testing. International Organization for Standardization, Geneva.

International Organization for Standardization, 2000a. ISO 3308. Routine Analytical Cigarette-smoking Machine. Definitions and Standard Conditions. International Standards Organization, Geneva.

International Organization for Standardization, 2000b. ISO 4387. Cigarettes – Determination of Total and Nicotine-free Dry Particulate Matter Using a Routine Analytical Smoking Machine. International Organization for Standardization, Geneva.

International Organization for Standardization, 2000c. ISO 10315. Cigarettes – Determination of Nicotine in Smoke Condensates - Gas Chromatographic Method. International Organization for Standardization, Geneva.

International Organization for Standardization, 2002. ISO 9512. Cigarettes – Determination of Ventilation – Definitions and Measurement Principles. International Organization for Standardization, Geneva.

International Organization for Standardization, 2005. ISO 6565. Tobacco and Tobacco Products - Draw Resistance of Cigarettes and Pressure Drop of Filter Rods - Standard Conditions and Measurement. International Organization for Standardization, Geneva.

International Organization for Standardization, 2009. ISO 2965. Materials Used as Cigarette Papers, Filter Plug Wrap and Filter Joining Paper, Including Materials Having a Discrete or Oriented Permeable Zone and Materials with Bands of Differing Permeability – Determination of Air Permeability. International Organization for Standardization, Geneva.

Lam, D.C., Luo, S.Y., Fu, K.H., Lui, M.M., Chan, K.H., Wistuba, I.I., Gao, B., Tsao, S.W., Ip, M.S., Minna, J.D., 2016. Nicotinic acetylcholine receptor expression in human airway correlates with lung function. Am. J. Physiol. Lung Cell Mol. Physiol. 310, L232–L239.

Life Sciences Research Office, 2004. Review of Ingredients Added to Cigarettes. Phase Two: Scientific Criteria for the Evaluation of Ingredients Added to Cigarettes. LSRO, Bethesda, MD.

Maneckjee, R., Minna, J.D., 1994. Opioids induce while nicotine suppresses apoptosis in human lung cancer cells. Cell Growth Differ. 5, 1033–1040.

Marks, M.J., Stitzel, J.A., Collins, A.C., 1985. Time course study of the effects of chronic nicotine infusion on drug response and brain receptors. J. Pharmacol. Exp. Ther. 235, 619–628.

Maron, D.M., Ames, B.N., 1983. Revised methods for the Salmonella mutagenicity test. Mutat. Res. 113, 173–215.

Morgan, E.D., 2000. Supercritical Fluid Extraction. . https://www.thevespiary.org/library/Files_Uploaded_by_Users/Sedit/Chemical%20Analysis/Encyclopedia%20of%20Separation%20Science/Level%20III%20-%20Practical%20Applications/NATURAL%20PRODUCTS%20-%20Supercritical%20Fluid%20Extraction.pdf (accessed 29-Dec-2015).

Naik, P., Fofaria, N., Prasad, S., Sajja, R.K., Weksler, B., Couraud, P.O., Romero, I.A., Cucullo, L., 2014. Oxidative and pro-inflammatory impact of regular and denicotinized cigarettes on blood brain barrier endothelial cells: is smoking reduced or nicotine-free products really safe?. BMC Neurosci. 15, 51.

O'Connor, R.J., Hammond, D., McNeill, A., King, B., Kozlowski, L.T., Giovino, G.A., Cummings, K.M., 2008. How do different cigarette design features influence the standard tar yields of popular cigarette brands sold in different countries?. Tob. Control 17 (Suppl. 1), i1–i5.

Oldham, M.J., Haussmann, H.J., Gomm, W., Rimmer, L.T., Morton, M.J., McKinney Jr., W.J., 2012. Discriminatory power of standard toxicity assays used to evaluate ingredients added to cigarettes. Regul. Toxicol. Pharmacol. 62, 49–61.

Organisation for Economic Co-operation and Development, 1997a. No. 471. Bacterial Reverse Mutation Test. OECD, Paris.

Organisation for Economic Co-operation and Development, 1997b. No. 432. In Vitro 3T3 NRU Phototoxicity Test. OECD, Paris.

Patskan, G.J., Podraza, K.F., Meurrens, K., Coggins, C.R.E., Friedrichs, B., Gerstenberg, B., Gomm, W., Schnell, P., Stabbert, R., Veltel, D., Weber, S., Terpstra, P.M., 2008. Toxicological comparisons of three styles of a commercial U.S. cigarette (Marlboro®) with the 1R4F reference cigarette. Inhal. Toxicol. 20, 695–721.

Piade, J.J., Wajrock, S., Jaccard, G., Janeke, G., 2013. Formation of mainstream cigarette smoke constituents prioritized by the World Health Organization–yield patterns observed in market surveys, clustering and inverse correlations. Food Chem. Toxicol. 55, 329–347.

Prasad, R., Grubbs, H.J., 1996. Process and apparatus for the semicontinuous extraction of nicotine from tobacco. U.S. Patent No. 5,497,792. <http://www.google.com/patents/US5497792 (accessed 29-Dec-2015).

Putnam, K.P., Bombick, D.W., Doolittle, D.J., 2002. Evaluation of eight in vitro assays for assessing the cytotoxicity of cigarette smoke condensate. Toxicol. In Vitro 16, 599–607.

Rickert, W.S., Trivedi, A.H., Momin, R.A., Wright, W.G., Lauterbach, J.H., 2007. Effect of smoking conditions and methods of collection on the mutagenicity and cytotoxicity of cigarette mainstream smoke. Toxicol. Sci. 96, 285–293.

Roemer, E., Tewes, F.J., Meisgen, T.J., Veltel, D.J., Carmines, E.L., 2002. Evaluation of the potential effects of ingredients added to cigarettes. Part 3: In vitro genotoxicity and cytotoxicity. Food Chem. Toxicol. 40, 105–111.

Roemer, E., Stabbert, R., Rustemeier, K., Veltel, D.J., Meisgen, T.J., Reininghaus, W., Carchman, R.A., Gaworski, C.L., Podraza, K.F., 2004. Chemical composition, cytotoxicity and mutagenicity of smoke from US commercial and reference cigarettes smoked under two sets of machine smoking conditions. Toxicology 195, 31–52.

Roemer, E., Dempsey, R., Hirter, J., Deger, E.A., Weber, S., Ode, A., Wittke, S., Schorp, M.K., 2014. Toxicological assessment of kretek cigarettes Part 6: the impact of ingredients added to kretek cigarettes on smoke chemistry and in vitro toxicity. Regul. Toxicol. Pharmacol. 70 (Suppl. 1), S66–S80.

Scherer, G., 1999. Smoking behaviour and compensation: a review of the literature. Psychopharmacology (Berl) 145, 1–20.

Shields, P.G., 2002. Tobacco smoking, harm reduction, and biomarkers. J. Natl. Cancer Inst. 94, 1435–1444.

Stephens, W.E., 2007. Dependence of tar, nicotine and carbon monoxide yields on physical parameters: implications for exposure, emissions control and monitoring. Tob. Control 16, 170–176.

Strasser, A.A., O'Connor, R.J., Mooney, M.E., Wileyto, E.P., 2006. Digital image analysis of cigarette filter stains as an indicator of compensatory smoking. Cancer Epidemiol. Biomark. Prev. 15, 2565–2569.

Thun, M.J., Henley, S.J., Calle, E.E., 2002. Tobacco use and cancer: an epidemiologic perspective for geneticists. Oncogene 21, 7307–7325.

U.S. Department of Health and Human Services, 1996. Good Laboratory Practice for Non-clinical Laboratory Studies. . 21 C.F.R. 58 http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=58 (accessed 29-Dec-2015).

Ungar, K., 1992. The INVITTOX data bank of in-vitro techniques in toxicology. Hum. Exp. Toxicol. 11, 151–154.

US Food and Drug Administration Center for Tobacco Products, 2012. Harmful and potentially harmful constituents in tobacco products and tobacco smoke: established list. Fed. Regist. 77, 20034–20037.

Walker, N., Fraser, T., Howe, C., Laugesen, M., Truman, P., Parag, V., Glover, M., Bullen, C., 2014. Abrupt nicotine reduction as an endgame policy: a randomised trial. Tob. Control http://dx.doi.org/10.1136/tobaccocontrol-2014–051801.

Warren, G.W., Romano, M.A., Kudrimoti, M.R., Randall, M.E., McGarry, R.C., Singh, A.K., Rangnekar, V.M., 2012. Nicotinic modulation of therapeutic response in vitro and in vivo. Int. J. Cancer 131, 2519–2527.